Characterization of Chloramphenicol-Resistant *Haemophilus influenzae*

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We examined nine chloramphenicol-resistant (minimal inhibitory concentration, ≥15 µg/ml) *Haemophilus influenzae* strains isolated in various parts of the world to characterize the genetic and biochemical bases of the resistance; four were type b. All nine contained conjugative plasmids, ranging in molecular weight from 34×10^6 to 46×10^6, which encoded for resistance to chloramphenicol and tetracycline or chloramphenicol, tetracycline, and ampicillin. Deoxyribonucleic acid homology studies showed that these plasmids were closely related to a previously described ampicillin-resistant plasmid, RSF007, and to each other. All nine isolates and their chloramphenicol-resistant transconjugants produced chloramphenicol acetyltransferase. We conclude that chloramphenicol resistance in these strains of *H. influenzae* is via plasmid-mediated production of chloramphenicol acetyltransferase.

Haemophilus influenzae causes serious infections in children, notably, meningitis and epiglottitis. The emergence of ampicillin resistance in *H. influenzae* in 1974 and its spread through the population has necessitated the use of chloramphenicol in the initial antibiotic therapy of children with systemic *H. influenzae* infections. Unfortunately, in the last few years chloramphenicol-resistant *H. influenzae* organisms were isolated from both healthy and infected individuals. Since 1978, chloramphenicol-, tetracycline-, and ampicillin-resistant strains (Cm^r, Tc^r, Ap^r) have been isolated, particularly in children migrating from the Far East. These multiresistant *H. influenzae* strains present a serious problem in the treatment of disease due to such organisms.

Chloramphenicol resistance in *H. influenzae* has not been well characterized, although Zaidenzaig et al. (15) examined a chloramphenicol acetyltransferase (CAT) partially purified from a chloramphenicol-resistant *H. parainfluenzae* isolate. In this study, we characterized nine chloramphenicol-resistant *H. influenzae* strains isolated in various parts of the world from both healthy and infected people. CAT activity was found in each of the strains.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1.

Media. The solid medium used for the growth of *H. influenzae* was a modified Kellogg agar plate supplemented with 10 µg of hemin, 10 µg of L-histidine, and 2 µg of nicotinamide adenine dinucleotide per ml (2). For certain experiments, the solid medium was

supplemented with 5 µg of chloramphenicol, 5 µg of tetracycline, 10 µg of ampicillin, 10 µg of rifampin, 10 µg of erythromycin, or 250 µg of streptomycin per ml; drugs were added singly or two in combination. Brain heart infusion, 3.5% (Difco), was used to grow *Escherichia coli*. The same nutrient broth supplemented with 10 µg of hemin, 10 µg of L-histidine, and 2 µg of nicotinamide adenine dinucleotide per ml was used for *H. influenzae*. Chloramphenicol-resistant *E. coli* strains were also grown on MacConkey agar containing 20 µg of chloramphenicol per ml. Plate cultures were grown at 36.5°C in 5% CO_2, whereas liquid cultures were incubated at 37°C and shaken at 200 rotations per min.

Agarose gel electrophoresis. Cleared lysates of bacterial strains were prepared as described by Meyers et al. (9). Strains were also lysed by using the procedure described by Hansen and Olsen (4). Both were subjected to electrophoresis through a 0.7% agarose gel. The molecular mass of each plasmid was calculated from the distance traveled down the gel compared with molecular weight standards derived from *E. coli* (9).

Determinations of minimal inhibitory concentrations. The minimal inhibitory concentrations were determined by agar dilutions, using a Steers replicator. An inoculum of 10^5 was used as described by Syriopoulou et al. (13).

Mating procedure. Filter matings were performed by using a modification of the method of van Klingeren et al. (14). Donor and recipient strains were grown separately to a density of 10^8 per ml. Equal volumes of donor and recipient were placed on a Bio-Rad 0.2-µm filter and incubated overnight. After incubation, the cells were rinsed from the filter, diluted, and plated. The recipient strain, *H. influenzae* Rd Rif^r Ery^r Str^r (rifampin resistant, erythromycin resistant, and streptomycin resistant), was mated with each chloramphenicol-resistant *H. influenzae* strain. All donors

TABLE 1. *Bacterial strains and their plasmids*

Strain	Plasmid	Date iso-lated (yr)	Geographic area	Isolation site	Capsule type	Bio-type	Resistance[a]
H. influenzae							
HC234	pRI234	1976	The Netherlands	Pharynx		II	Cm Tc
R328	pMR328	1976	France	CSF[b]	Non-b[c]	II	Cm Tc
77-36098	pMR7736	1977	United States	Blood	b	III	Cm Tc
R374	pMR374	1978	Canada	Sputum		II	Cm Tc Ap
R375	pMR375	1978	United States	Trachea	b	II	Cm Tc Ap
9-80118	pMR9801	1979	United States	Ear		II	Cm Tc
R384	pMR384	1979	United States	Pharynx		II	Cm Tc
R385	pMR385	1979	United States	CSF	b	I	Cm Tc Ap
R387	pMR387	1979	Thailand	CSF	b	II	Cm Tc Ap
HR7	RSF007	1975	United States		b		Ap
Rd		1944	United States		d[d]		Rif Str Ery
E. coli							
J53							
J53	R1						Ap Sm Cm (type I) Km Su
J53	Sa						Sm Cm (type II) Km Su
J53	R387						Sm Cm (type III)

[a] Plasmid-mediated resistance: Cm, chloramphenicol; Ap, ampicillin; Tc, tetracycline; Sm, streptomycin; Su, sulfonamide; Km, kanamycin. Chromosomal resistance: Rif, rifampin; Str, streptomycin; Ery, erythromycin.

[b] CSF, Cerebrospinal fluid.

[c] R328 was originally a capsulated strain. The strain has lost its capsule, and we were unable to determine its type.

[d] Rd is a type d strain which no longer produces a detectable capsule.

were rifampin susceptible and erythromycin or strep-tomycin susceptible or both. Transconjugants were selected on 5 μg of tetracycline and 10 μg of rifampin, 5 μg of chloramphenicol and 10 μg of rifampin, or 10 μg of ampicillin and 10 μg of rifampin per ml. The numbers of donors and recipients were calculated by plating on media containing chloramphenicol or rif-ampin. Plates were incubated overnight, and individ-ual transconjugants were selected. A minimum of three matings were performed with each donor, and the mean frequency of transfer was calculated.

Testing antibiotic resistance of transconju-gants. Individual transconjugants were restreaked onto the same selective medium two to four times. Inocula for antibiotic susceptibility testing were re-moved from selective plates after overnight incuba-tion. Cells were suspended in buffered saline and di-luted to allow the Steers replicator to deliver between 10^4 and 10^5 colony-forming units to the test plate. Both donor and recipient controls were included in each run. The antibiotic (Rif, Str, Ery, Cm, Tc, Ap) plates were inoculated first, followed by an antibiotic-free plate; only isolates which were Rif^r Str^r Ery^r were counted. This eliminated any donors which had mu-tated to rifampin resistance. Between 50 and 100 col-onies were tested for each resistance marker. The isolates from a minimum of three different matings were tested for antibiotic susceptibility.

Preparation of plasmid DNA. [^{3}H]thymidine-la-beled plasmid deoxyribonucleic acid (DNA) was pre-pared from *H. influenzae* HR7 and HC234 as previ-ously described by De Graaff et al. (1).

DNA-DNA hybridization studies. Unlabeled whole-cell DNA from *H. influenzae* was prepared by the method of So et al. (12). Hybridizations were done at 63°C for 21 h and assayed by the S₁ endonuclease technique (12).

CAT and characterization. CAT-containing ex-tracts were prepared from nine Cm^r *H. influenzae* strains, Cm^s *H. influenzae* Rd, Cm^r transconjugants, *E. coli* J53 carrying plasmids R1, Sa, and R387, and Cm^s R$^-$ *E. coli* J53 from late-log-phase cells as de-scribed by Zaidenzaig and Shaw (16). CAT activity was assayed spectrophotometrically by determining the chloramphenicol-dependent appearance of coen-zyme A, using acetyl-coenzyme A as the substrate. Protein concentrations were determined by the method of Lowry et al. (8), using bovine serum albu-min as the standard.

RESULTS

Demonstration of plasmids in chloram-phenicol-resistant *H. influenzae*. Cleared ly-sates of each chloramphenicol-resistant strain were electrophoresed on a 0.7% agarose gel (Fig. 1); with the exception of slot I, each strain ap-peared to carry a single large plasmid. (Slot A has molecular weight standards.) Strain R387 from Thailand (slot I) carried a large plasmid and two smaller ones. Molecular weights for the large plasmids determined from agarose gels ranged between 34×10^6 and 46×10^6.

Relationships between plasmids from *H. influenzae*. Previously, a number of large R plasmids from *H. influenzae* bearing different resistance genes have been studied (3, 6, 7, 10, 14). The group as a whole appears to share at least 60% of their polynucleotide sequences with each other, suggesting that they belong to the same incompatibility group or that these orga-nisms carry a common core plasmid to which
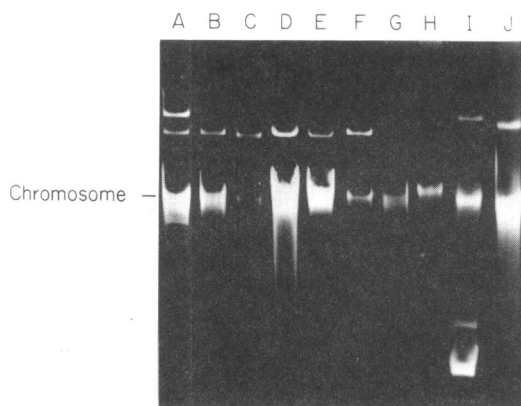
FIG. 1. *Agarose gel electrophoresis of DNA from cleared lysates of Cm^r H. influenzae. (A) Molecular weight standards ranging from 62 × 10^6 to 4.2 × 10^6; (B) strain HC234; (C) strain 9-80118; (D) strain R328; (E) strain R374; (F) strain R384; (G) strain R385; (H) strain R375; (I) strain R387; (J) strain 77-36098.*

resistance genes have been translocated (1-3, 5). To determine whether these Cm^r plasmids share DNA sequences with each other and with other previously isolated R plasmids, we performed hybridization experiments. Plasmids RSF007, isolated in 1975 from *H. influenzae* HR7 and carrying the TEM β-lactamase gene, and pRI234, isolated in 1976 and carrying Cm^r and Tc^r determinants, were chosen as probes for the study; each has been well characterized (1-3, 14). [^{3}H]thymidine-labeled RSF007 plasmid DNA, [^{3}H]thymidine-labeled pRI234 plasmid DNA, and unlabeled whole-cell DNA from each of the chloramphenicol-resistant isolates and the appropriate control strains were tested (Table 2). Plasmid pRI234 appeared to share 60 to 93% of its DNA sequences with the other chloramphenicol-resistant plasmids. The ampicillin-resistant plasmid RSF007 shared 61 to 89% of its sequences with the chloramphenicol-resistant plasmids; these data suggest that all of these plasmids share a common ancestor.

Transferability and kinetics of resistance gene transfer. Filter matings were performed to determine whether the plasmids were capable of conjugal transfer. The chloramphenicol-resistant strains were all susceptible to rifampin and streptomycin and/or erythromycin. A laboratory *H. influenzae* strain, Rd, resistant to rifampin, streptomycin, and erythromycin was used as the recipient. The transconjugants were selected on plates containing chloramphenicol and rifampin, tetracycline and rifampin, or ampicillin and rifampin. Colonies were then tested for growth on streptomycin and erythromycin. Only those colonies which were rifampin, strep-

tomycin, and erythromycin resistant were used. Fifty to 100 transconjugants from each medium were screened for unselected markers (Cm, Tc, and Ap). Selected transconjugants were lysed; the resistance to Tc, Cm, and Ap was always correlated with the presence of a 34- to 46-megadalton plasmid. The data from three sets of experiments are summarized in Table 3. All of the plasmids, with the exception of pMR387, transferred Tc and Cm or Tc, Cm, and Ap as a unit at a frequency ranging from 10^{-3} to 10^{-5}. On occasion, some of the transconjugants were susceptible to tetracycline or ampicillin. Over 1,000 transconjugants were examined; none were found to have lost more than one resistance determinant or to be susceptible to chloramphenicol.

Plasmid pMR387 appeared to be different from the others. This plasmid is from the Thailand strain, which carries at least three different plasmid species. When strain R387 was used as a donor, the Tc and Cm determinants appeared to transfer as a unit. However, the ampicillin determinant did not. In the first mating experiment ampicillin transferred as an unselected marker 40 to 47% of the time with Cm or Tc. However, in subsequent mating, the frequency of cotransfer of ampicillin and Tc or Cm was considerably less, yielding an average for three matings of 7 to 14%. The first matings were performed soon after we received the strain, whereas the others were performed 1 and 2 months later. However, the reduced frequency

TABLE 2. *Hybridization between ^{3}H-labeled RSF007 and ^{3}H-labeled pRI234 plasmid DNA and unlabeled whole-cell DNA*

H. influenzae strain[a]	Relative DNA sequence homology (%) with plasmid DNA[b]	
	RSF007	pRI234
HR7 (RSF007)	100	61
HC234 (pRI234)	69	100
R328	73	86
77-36098	71	71
R374	89	82
R375	79	93
9-80118	77	65
R384	61	60
R385	89	71
R387	66	79
Rd	1	<1

[a] Source of unlabeled whole-cell DNA.

[b] The actual extent of binding of ^{3}H-labeled RSF007 and pRI234 plasmid DNA with whole-cell DNA of their respective parental strain was 70 to 75%. All other reactions were normalized to these values, taken as 100%. Each value shown is the average of three reactions.

TABLE 3. *Molecular weights and transfer frequencies of Cm^r plasmids*

Plasmid	Mol wt (×10^6)	Frequency of plasmid transfer/donor	Selected marker	Antibiotic transfer		
				Unselected marker (%)		
				Cm	Tc	Ap
pR1234	38	2 × 10^{-3}	Cm	—[a]	90	—
			Tc	100	—	
pMR328	37	8 × 10^{-4}	Cm	—	75	
			Tc	100	—	
pMR7736	40	4 × 10^{-5}	Cm	—	100	
			Tc	100	—	
pMR9801	35	6 × 10^{-4}	Cm	—	82	
			Tc	100	—	
pMR384	35	3 × 10^{-5}	Cm	—	100	
			Tc	100	—	
pMR374	34	6 × 10^{-5}	Cm	—	98	100
			Tc	100	—	100
			Ap	100	96	—
pMR375	46	6 × 10^{-5}	Cm	—	100	100
			Tc	100	—	100
			Ap	100	100	—
pMR385	38	2 × 10^{-4}	Cm	—	83	97
			Tc	100	—	100
			Ap	100	83	—
pMR387	42	2 × 10^{-5}	Cm	—	98	14 (47)[b]
			Tc	100	—	13 (41)[b]
			Ap	7	7	—

[a] —, Not applicable.

[b] In the first mating, Ap transferred as an unselected marker with Cm at 47% and with Tc at 41%. In later matings, cotransfer was significantly reduced, and the average cotransfer was 14 or 13%.

of cotransfer of ampicillin was also found in the frozen stock of this strain. Since the strain carries at least three plasmids, the ampicillin determinant may have been on one of the smaller plasmids. Transconjugants derived from this strain, two resistant to ampicillin and the other resistant to chloramphenicol and tetracycline, along with the donor R387, which had lost its resistance to ampicillin, were examined by agarose gel electrophoresis. All transconjugants showed a single 42-megadalton plasmid band (Fig. 2).

In addition, the plasmid complement seen in the Cm^r Tc^r Ap^s donor (R387) was identical to that of the R387 Cm^r Tc^r Ap^r donor (Fig. 1). This suggests that the ampicillin determinant is not on one of the small plasmids. Twenty R387 transconjugants selected for Cm or Ap were subsequently lysed; none had received the smaller plasmids.

Characterization of CAT. Chloramphenicol resistance is commonly associated with the presence of the enzyme CAT. Three different plasmid-mediated enzymes have been characterized among the *Enterobacteriaceae* (types I, II, and III). A type III-like enzyme has been described in a chloramphenicol-resistant *H. parainfluenzae* strain (15). Therefore, we wanted to de-

termine whether these chloramphenicol-resistant *H. influenzae* strains produced CAT.

Enzyme extracts were prepared from each *H. influenzae* strain and a corresponding transconjugant along with the *E. coli* strains carrying one of the prototypic enzymes. Activity was assayed with acetyl-coenzyme A, chloramphenicol, and the supernatant derived from sonicated bacteria and monitored spectrophotometrically. *H. influenzae* Rd and *E. coli* J53, both susceptible to chloramphenicol, did not have detectable CAT activity after 60 min of incubation at 37°C. All of the resistant strains had measurable activity (Table 4).

With the exception of strain R387, all of the clinical strains and the corresponding transconjugants showed essentially identical CAT activity within the limit of the assay's sensitivity (±0.25 µmol/min per mg). Thus, it appears that all of the chloramphenicol-resistant *H. influenzae* strains and the corresponding transconjugant strains do have measurable CAT activity which is capable of being transferred by conjugation.

DISCUSSION

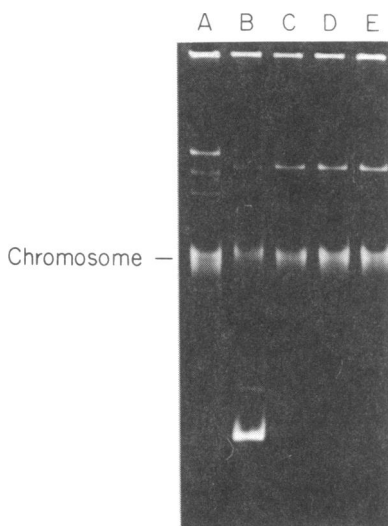The nine chloramphenicol-resistant *H. influenzae* strains examined contained conjugative

FIG. 2. *Agarose gel electrophoresis of DNA from cleared lysates of H. influenzae. (A) Molecular weight standards ranging from 62 × 10⁶ to 23 × 10⁶; (B) donor R387 Cmʳ Tcʳ Apˢ containing three plasmid species; (C and D) Apʳ Cmˢ Tcˢ transconjugants carrying a 42-megadalton plasmid band; (E) Apˢ Cmʳ Tcʳ transconjugants carrying a 42-megadalton plasmid band.*

Chromosome —

TABLE 4. *CAT activity*

Strain	Cm MIC[a] (µg/ml)	CAT activity[b] (µmol/min per mg)	CAT activity[b] of Rd trans-conjugant (µmol/min per mg)
H. influenzae			
Rd	1	<0.05[c]	
9-80118	15	0.76	0.81
R374	15	0.90	0.47
R384	15	0.36	0.37
R328	20	0.57	0.31
HC324	25	0.56	0.81
77-36098	25	0.45	0.85
R385	25	0.28	0.41
R375	40	3.30	3.23
R387	40	2.77	0.37
E. coli			
J53	Cmˢ	<0.05	
J53 (R1)	Cmʳ	2.89	
J53 (Sa)	Cmʳ	0.56	
J53 (R387)	Cmʳ	2.79	

[a] Chloramphenicol minimal inhibitory concentrations (MICs) determined with a 10⁵ inoculum by agar dilution.

[b] CAT activity ± 0.25 µmol/min per mg.

[c] <0.05 µmol/min per mg detected during a 60-min incubation.

plasmids encoding resistance to chloramphenicol and tetracycline or chloramphenicol, tetracycline, and ampicillin. With the exception of the strain from Thailand, all resistance markers were generally transferred as a single unit. van Klingeren et al. (14) studied the first chloramphenicol- and tetracycline-resistant *H. influenzae* clinical isolate, HC234. Over 10,000 transconjugants were selected for tetracycline and screened for chloramphenicol resistance. No chloramphenicol-susceptible colonies were isolated. Similarly, we have examined over 1,000 transconjugant colonies derived from eac'₊ strain, and except for strain R387, we were unable to find chloramphenicol-susceptible transconjugants. Therefore, with the exception of the Thailand strain, each resistant strain usually cotransfers the chloramphenicol and tetracycline resistance determinants.

Strain R387 is unusual in that it carries at least three different plasmid species. However, like the other strains, R387 transfers chloramphenicol and tetracycline as a single unit. The ampicillin determinant initially transferred with these two markers at only 40%. The ability to transfer ampicillin at even this frequency was quickly lost. Transfer of ampicillin resistance itself was reduced 100-fold in comparison with chloramphenicol and tetracycline resistance; with some clones the ability to transfer ampicillin resistance was lost, but the strain remained resistant to ampicillin. The ampicillin marker appears to be carried on a large plasmid of the same molecular weight as the tetracycline and chloramphenicol markers. Whether R387 actually carries two large plasmids of similar molecular weight or one plasmid carries all three determinants is still under investigation.

The transfer of Cmʳ is correlated with the presence of CAT activity. The two clinical *H. influenzae* strains, R375 and R387, which have the highest minimal inhibitory concentrations for chloramphenicol (40 µg/ml) by agar dilution also appear to have more CAT activity than the other chloramphenicol-resistant *H. influenzae* strains. This higher level of enzyme activity is transferred to the transconjugants when R375 is the donor but not when R387 is the donor. R387 may have more than one determinant for chloramphenicol resistance; this is under investigation. However, there appears to be no correlation between agar minimal inhibitory concentrations and measurable CAT activity with the other resistant strains.

At the present time, the CATs from these strains are being characterized to determine whether they are similar to one of the three enteric plasmid-mediated enzymes previously

described or whether they represent a new variant.

LITERATURE CITED

1. De Graaff, J., L. P. Elwell, and S. Falkow. 1976. Molecular nature of two beta-lactamase-specifying plasmids isolated from *Haemophilus influenzae* type b. J. Bacteriol. **126**:439–446.

2. Elwell, L. P., J. De Graaff, D. Seibert, and S. Falkow. 1975. Plasmid-linked ampicillin resistance in *Haemophilus influenzae* type b. Infect. Immun. **12**:404–410.

3. Elwell, L. P., J. R. Saunders, M. H. Richmond, and S. Falkow. 1977. Relationships among some R plasmids found in *Haemophilus influenzae*. J. Bacteriol. **131**: 356–362.

4. Hansen, J. B., and R. H. Olsen. 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.

5. Jahn, G., R. Laufs, P. M. Kaulfers, and H. Kolenda. 1979. Molecular nature of two *Haemophilus influenzae* R factors containing resistances and the multiple integration of drug resistance transposons. J. Bacteriol. **138**:584–597.

6. Kaulfers, P. M., R. Laufs, and G. Jahn. 1978. Molecular properties of transmissible R factors of *Haemophilus influenzae* determining tetracycline resistance. J. Gen. Microbiol. **105**:243–252.

7. Laufs, R., and P. M. Kaulfers. 1977. Molecular characterization of a plasmid specifying ampicillin resistance and its relationship to other R factors from *Haemophilus influenzae*. J. Gen. Microbiol. **103**:277–286.

8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

9. Meyers, J. A., D. Sanches, L. P. Elwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.

10. Saunders, J. R., L. P. Elwell, S. Falkow, R. B. Sykes, and M. H. Richmond. 1978. β-Lactamases and R-plasmids of *Haemophilus influenzae*. Scand. J. Infect. Dis. Suppl. **13**:16–22.

11. Shaw, W. V. 1967. The enzymatic acetylation of chloramphenicol by extracts of R factor-resistant *Escherichia coli*. J. Biol. Chem. **242**:687–693.

12. So, M., J. H. Crosa, and S. Falkow. 1975. Polynucleotide sequence relationships among Ent plasmids and the relationship between Ent and other plasmids. J. Bacteriol. **121**:234–238.

13. Syriopoulou, V. P., D. W. Scheifele, C. M. Sack, and A. L. Smith. 1979. Effect of inoculum size on the susceptibility of *Haemophilus influenzae* b to beta-lactam antibiotics. Antimicrob. Agents Chemother. **16**: 510–513.

14. van Klingeren, B., J. D. A. van Embden, and M. Dressens-Kroon. 1977. Plasmid-mediated chloramphenicol resistance in *Haemophilus influenzae*. Antimicrob. Agents Chemother. **11**:383–387.

15. Zaidenzaig, Y., J. E. Fitton, L. C. Packmand, and W. V. Shaw. 1979. Characterization and comparison of chloramphenicol acetyltransferase variants. Eur. J. Biochem. **100**:609–618.

16. Zaidenzaig, Y., and W. V. Shaw. 1976. Affinity and hydrophobic chromatography of three variants of chloramphenicol acetyltransferases specified by R factors in *Escherichia coli*. FEBS Lett. **62**:266–271.